Decreased PPAR-γ expression in the conjunctiva and increased expression of TNF-α and IL-1β in the conjunctiva and tear fluid of dry eye mice

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Abstract. The aim of this study was to investigate the expression of peroxisome proliferator-activated receptor γ (PPAR-γ), tumor necrosis factor (TNF)-α and interleukin (IL)-1β in the conjunctiva and the association between inflammatory cytokines and PPAR-γ in dry eye mice. Dry eye was induced in 6-week-old female C57 mice. mRNA expression of PPAR-γ, TNF-α and IL-1β were measured. PPAR-γ protein expression in the conjunctiva, and the contents of TNF-α and IL-1β in the conjunctiva and tear-wash fluid were determined. A PPAR-γ agonist, pioglitazone (PIO), was used to treat dry eye mice. Dry eye mice presented with similar manifestations as in humans. The PPAR-γ expression in the conjunctiva of dry eye mice was downregulated, accompanied by increased contents of TNF-α and IL-1β. PIO treatment markedly reduced the contents of TNF-α and IL-1β in tear fluid of dry eye mice. Following PIO treatment, the PPAR-γ expression increased markedly. PIO may activate PPAR-γ to inhibit the expression of the inflammatory cytokines TNF-α and IL-1β in dry eye mice. This suppresses the inflammatory progression, increases the tear fluid production, elevates the tear film stability and reduces the damage to the ocular surface, exerting a therapeutic effect on dry eye.

Introduction

Dry eye is defined by the International Dry Eye Workshop as a multifactorial disease of the tear fluid and ocular surface that results in symptoms of discomfort, visual disturbance and tear film instability, with potential damage to the ocular surface. It is usually accompanied by increased osmolarity of the tear film and inflammation of the ocular surface (1).

There is increasing evidence demonstrating that inflammation is important in the pathogenesis of dry eye. Whatever the initial cause of dry eye is, during the development of this disease, a vicious cycle of inflammation may develop in the ocular surface, which leads to ocular surface destruction. Continuous dryness of the ocular surface may result in excessive nervous stimulation leading to neurogenic inflammation, lymphocytic infiltration and increased immune reactivity, and release of inflammatory cytokines into the lacrimal glands, tear fluid and conjunctiva. In addition, inflammatory mediators may inhibit the neural signals to the lacrimal gland, which worsens the ocular surface desiccation and, simultaneously, destroys the ocular surface environment leading to tissue destruction. A previous study has shown that a number of inflammatory cytokines, including interleukin (IL)-6, IL-1 and tumor necrosis factor-α (TNF-α), increase in the tear fluid of dry eye patients and the expression of these inflammatory cytokines are also upregulated in the corneal epithelium and conjunctiva of dry eye patients and animals (2).

Peroxisome proliferator-activated receptor γ (PPAR-γ) is a member of the ligand-activated nuclear receptor superfamily and has been extensively studied in adipocytes, where it is key in the glucose homeostasis and adipocyte differentiation (3). In the last decade, PPAR-γ has been found to possess anti-inflammatory activity (4). There is increasing evidence suggesting that PPAR-γ exhibits an anti-inflammatory role by negatively regulating the expression of pro-inflammatory cytokines activated during the inflammatory response. PPAR-γ acts as a heterodimer with retinoid X receptors to regulate gene expression by recognizing and binding to PPAR response elements located in the promoters of target genes (5). PPAR-γ interferes with the inflammatory response at different levels by modulating the expression of inflammatory mediators. Following binding to ligands, PPAR-γ may inhibit the expression of inducible nitric oxide synthase (iNOS) and MMP-9 and the production of TNF-α, IL-6 and IL-1β (6). It has been well

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documented that PPAR-γ and PPAR-γ ligands are beneficial for airway inflammation (7) and inflammatory bowel disease in various animal models (8). The exact mechanisms by which PPAR-γ regulates the production of inflammatory cytokines are complicated and not fully understood. There is evidence showing that PPAR-γ activation may inhibit the transcriptional activity of cytokine induced pro-inflammatory transcription factors, including activator protein-1 and nuclear factor (NF)-κB (9). These transcription factors are key in the expression of inflammatory cytokines.

PPAR-γ expression is tissue dependent. On the ocular surface, PPAR-γ is expressed in the lacrimal gland (10) and cornea (11) at a relatively low level. The conjunctiva contains mucosa-associated lymphoid tissue and shares a number of common features with the mucous membrane in the airway and digestive tract. The conjunctiva has a rich blood supply, and may be a target of inflammation at the early stages of the immune reaction during the development of dry eye pathology. PPAR-γ is hypothesized to be important in the inflammatory process during the pathogenesis of dry eye. To date, little is known concerning PPAR-γ expression in the conjunctiva, and to the best of our knowledge no studies have been undertaken to evaluate the change in PPAR-γ expression on the ocular surface in dry eye. The current study aimed to investigate the expression of PPAR-γ and inflammatory cytokines in the conjunctiva of dry eye mice to explore the role of PPAR-γ in the pathogenesis of dry eye.

Materials and methods

Dry eye mouse model. A total of 96 6-week-old female C57 BL/6J mice (SLAC Laboratory Animal Center, Shanghai, China) were randomly divided into eight groups, in which mice were sacrificed by cervical dislocation at eight different time points, between 1 and 20 days, following dry eye induction. A total of 12 mice per group were used for the detection of aqueous tear production and corneal fluorescence staining; 3 mice per group were used for the conjunctival histopathology, detection of PPAR-γ, IL-1β and TNF-α mRNA expression by quantitative polymerase chain reaction (qPCR), western blot analysis and concentration of IL-1β and TNF-α in tear fluid and the conjunctiva; and 75 mice were used for the treatment with pioglitazone (PIO). All experiments were performed in accordance with the Declaration of Helsinki or the NIH statement for Use of Animals in Research. The study was approved by the Ethics Committee of Tongji University (Shanghai, China). Animals were cared for in accordance with Statement for the Use of Animals in Ophthalmic and Vision Research of The Association of Research for Vision and Ophthalmology.

Dry eye was induced as previously reported by subcutaneous injection of 0.1 mg/0.2 ml scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA) in the hindquarters of the mice three times daily (9, 12 am and 5 pm). Animals were exposed to air draft for 12 h every day to maintain the ambient humidity below 40% (12).

Detection of aqueous tear production. The aqueous tear production was measured using the phenol-red impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA, USA). The threads were held with jeweler forceps (Katena Products, Inc., Denville, NJ, USA) and placed in the lateral cantus of the conjunctival fornix of the right eye for 60 sec (13). The length of moist thread was observed under a microscope (Olympus, Tokyo, Japan), using a slide gauge with a precision accuracy of 0.02 mm.

Corneal fluorescence staining. Corneal fluorescence staining was performed to evaluate the corneal integrity. Briefly, 0.5 µl of 1% flourescein (Sigma-Aldrich) was added to the inferior conjunctival sac of the right eye with a micropipette. The cornea was examined under a slit lamp biomicroscope (66 Vision Tech Co., Ltd., Suzhou, China) in cobalt blue light 1 min after fluorescein addition. Corneal fluorescein staining was classified using a grading system developed by Park *et al* (13) on the basis of area of corneal staining. No staining was graded as 0; stained area ≤1/8 of the cornea was graded as 1; stained area ≤1/4 of the cornea was graded as 2; stained area ≤1/2 of the cornea was graded as 3; and stained area >1/2 of the cornea was graded as 4.

Conjunctival histopathology. Mice were sacrificed by cervical dislocation and the eyeball containing conjunctiva was collected, fixed in 4% paraformaldehyde and embedded in paraffin. The eyes were sectioned in vertical plane (4-µm thick). These sections were stained using the periodic acid-Schiff (PAS) staining system (Sigma-Aldrich) according to the manufacturer's instructions. The morphology of the conjunctiva was observed and the goblet cells were counted under a microscope (Olympus) by two investigators blinded to the study. Three sections were collected from each sample for cell counting, and an average was calculated.

Detection of PPAR-γ, IL-1β and TNF-α mRNA expression by qPCR. Mice were sacrificed by cervical dislocation. Conjunctiva was harvested, immediately frozen in liquid nitrogen and stored at -80˚C until RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and 2 µg total RNA was reverse transcribed using the ReverTra Ace™ RT-PCR kit (Toyobo, Osaka, Japan) according to manufacturer's instructions. Following reverse transcription, 2 µl cDNA was used as template in 30 µl PCR mixture and *Taq* platinum. Primers were designed using DNA Star software (DNAStar, Inc., Madison, WI, USA) according to the manufacturer's instructions. Table I shows the primers used and the anticipated length of products.

SYBR-Green qPCR was performed in a Shanghai Hongshi Medical Technology Co, Ltd., Real-time PCR Detection system (Hongshi, Shanghai, China). In 30 µl SYBR-Green PCR reaction mixture, there were 2 µl cDNA, 1 µl forward primer, 1 µl reverse primer, 15 µl 2X PCR Mastermix (QPK-201; Toyobo, Osaka, Japan) and 11 µl PCR-grade water. For the detection of mRNA expression of PPAR-γ and TNF-α, the following conditions were used: 94˚C for 3 min, 36 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec. For the detection of mRNA expression of IL-1β the following conditions were used: 94˚C for 3 min for 1 cycle, 40 cycles of 94˚C for 20 sec and 60˚C for 40 sec. A negative control was included to evaluate DNA contamination. Products of qPCR were analyzed using a relative standard curve method with SLAN 5.0 software (Shanghai Hongshi Medical Technology

Table I. Primers used in PCR for amplification of target genes.

Gene	GeneBank no.	Sequence	Length (bp)
PPAR-γ	NM00112733	F: agtgccttgctgtggggatgt R: tcagcgggaaggactttatgtatg	180
TNF-α	NM013693	F: tgcaccaccatcaaggactcaaat R: ccccggccttccaaataaatacat	289
IL-1β	NM008361	F: actacaggctccgagatgaacaac R: cccaaggccacaggtatttt	144
GAPDH	NM008084	F: accacagtccatgccatcac R: tccaccaccctgttgctgta	450

PCR, polymerase chain reaction; PPAR-γ, peroxisome proliferator-activated receptor γ; TNF, tumor necrosis factor; IL, interleukin; F, forward; R, reverse.

Co., Ltd.). The mRNA expression of target genes were normalized to that of GAPDH.

Western blot analysis. The conjunctiva was collected and mixed in RIPA buffer, containing 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.01 mM tetrasodium pyrophosphate, 0.2 mM orthovanadate, 2 mM EDTA, 0.15 mol/l sodium chloride and 100 units proteinase cocktail, pH 7.2. Following denaturation, proteins were separated by 10% SDS polyacrylamide gel electrophoresis and transferred electronically to nitrocellulose membranes (Whatman 0.45 μm; 300 mA, 90 min; Bio-Rad, Hercules, CA, USA). Nonspecific binding was blocked with 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 (TBST) overnight at 4˚C. Next, the membranes were incubated with mouse anti-β-actin and mouse anti-PPAR-γ monoclonal primary antibody (1:1,000). The membranes were rinsed three times with TBST for 10 min. Next, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. The immunoreactive bands were visualized by enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA). The relative densities of target proteins were normalized against β-actin using a Gel-Pro analyzer (Media Cybernetics, Silver Spring, MD, USA).

Concentration of IL-1β and TNF-α in tear fluid and the conjunctiva. The concentration of IL-1β and TNF-α in the tear fluid and the conjunctiva were determined by ELISA. Tear-washing fluid was collected as described by Song *et al* (14). Mice in each group were divided into three subgroups (four per subgroup). The tear-washing fluids of the two eyes in the same subgroup were pooled together and stored at -80˚C until ELISA was performed. The conjunctiva was collected and added to 200 μl phosphate-buffered saline followed by homogenization. The homogenate was centrifuged at 35,000 x g for 20 min at 4˚C, and the supernatant was collected for ELISA. The pooled tear fluid was diluted (1:3), and the IL-1β and TNF-α concentration was measured with a ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All samples were assayed in duplicate and the means were calculated.

Treatment with PIO. Mice were divided into normal control, dry eye, 0.25% PIO, 0.5% PIO and 1% PIO groups. Dry eye was induced as described in the materials and methods, and treatment was performed 12 days later. PIO powder (Wuhan Huameihua Scientific Co., Ltd, Wuhan, China; 0.1 g) was dissolved in dimethylsulfoxide (DMSO) under aseptic conditions and diluted with normal saline tp 1, 0.5 and 0.25%. DMSO diluted with normal saline of equal volume served as a control. Treatment with PIO was performed three times daily on one of the eyes for 4 weeks. In the dry eye group, mice were treated with DMSO in normal saline. At the end of the experiment, the concentration of IL-1β and TNF-α in the tear fluid were measured by ELISA, and corneal fluorescein staining was performed. Mice were sacrificed by cervical dislocation, and the conjunctiva was collected. The mRNA and protein expression of PPAR-γ in the conjunctiva were determined with qPCR and western blot analysis, respectively, and PAS staining was performed to detect the goblet cell density.

Statistical analysis. Data are expressed as the mean ± standard deviation. Comparisons were performed using Student's-t test for independent samples. Statistical analysis was performed with SPSS version 16.0. (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Aqueous tear production. The aqueous tear production is shown in Table II. There was a significant decrease in aqueous tear production following the introduction of dry eye when compared with the control group ($P<0.001$). The tear production continued to decrease until 10-12 days following introduction of dry eye. Although there was a transient increase at 15 and 20 days, the tear production remained significantly lower compared with that of the control group.

Table II. Aqueous tear production, corneal fluorescein staining score and conjunctival goblet cell count of dry eye mice and control mice.

Variable	Aqueous tear production, mm; n=12	Corneal fluorescein staining score, n=12	Conjunctival goblet cell count, n=3
Control	5.66±0.66	0.11±0.33	31.00±2.64
Day 1	2.66±0.17[b]	1.22±0.44[b]	21.00±2.64[a]
Day 3	1.86±0.24[b]	2.33±0.50[b]	16.67±1.52[b]
Day 5	1.48±0.30[b]	2.67±0.50[b]	11.33±1.15[b]
Day 10	3.11±0.56[b]	3.33±0.70[b]	5.33±1.15[b]
Day 12	2.62±0.24[b]	3.78±0.44[b]	5.33±1.52[b]
Day 15	1.11±0.24[b]	3.89±0.33[b]	15.33±1.52[a]
Day 20	1.06±0.18[b]	4.00±0.00[b]	12.67±1.15[b]

[a]P<0.01 and [b]P<0.001, vs. the control group.

Corneal fluorescein staining. The grades of corneal fluorescein staining are shown in Table II. At 1 min following fluorescein treatment, no staining was observed in the corneas of the control mice. One day following the fluorescein treatment, scattered punctate staining was observed. Patches of punctate staining were observed five days following fluorescein treatment and diffuse corneal fluorescein staining was noted at 12 days following fluorescein treatment.

Conjunctival goblet cell count. The number of goblet cells in the conjunctiva of dry eye mice reduced gradually 1 day following introduction of dry eye. A significant difference was observed between dry eye mice and control mice at days 1-20 (P<0.01; Table II).

Downregulated PPAR-γ expression in the conjunctiva of dry eye mice. The PPAR-γ mRNA and protein expression in the conjunctiva were detected using qPCR and western blot analysis, respectively. Results showed the PPAR-γ mRNA expression was downregulated in dry eye mice as compared with control mice (P<0.01; Fig. 1A). Western blot analysis revealed a similar trend in the PPAR-γ mRNA expression (Fig. 1B and C).

Expression of TNF-α and IL-1β. The mRNA expression of TNF-α and IL-1β detected by qPCR is shown in Fig. 2A and B, respectively. The IL-1β mRNA expression increased markedly in the conjunctiva of dry eye mice as compared with control mice (P<0.01) except on day 15. The TNF-α mRNA expression increased between days 5 and 12 (P<0.05). ELISA showed that the TNF-α concentration of the conjunctiva (days 1-20) was: 2,006.33±165.79, 3,205.3±100.07, 3,225±102.34, 3,676.67±158.82, 4,675±76.02, 9,442.60±576.39 and 9,717±704.32 which was significantly higher compared with the control group (1,066.27±106.59; P<0.001; Fig. 2C). The TNF-α concentration in the tear fluid samples (days 1-20) was 2,609±68.57, 1,164.67±34.27, 1,158±36.37, 564.4±44.09, 2,276.33±183.02, 3,052.33±354.88 and 2,099.33±233.02 in dry eye groups, and 613±19.0 in control group (Fig. 2D). The IL-1β concentration of the conjunctiva (days 1-20) was 393.63±5.47, 407.47±16.4,

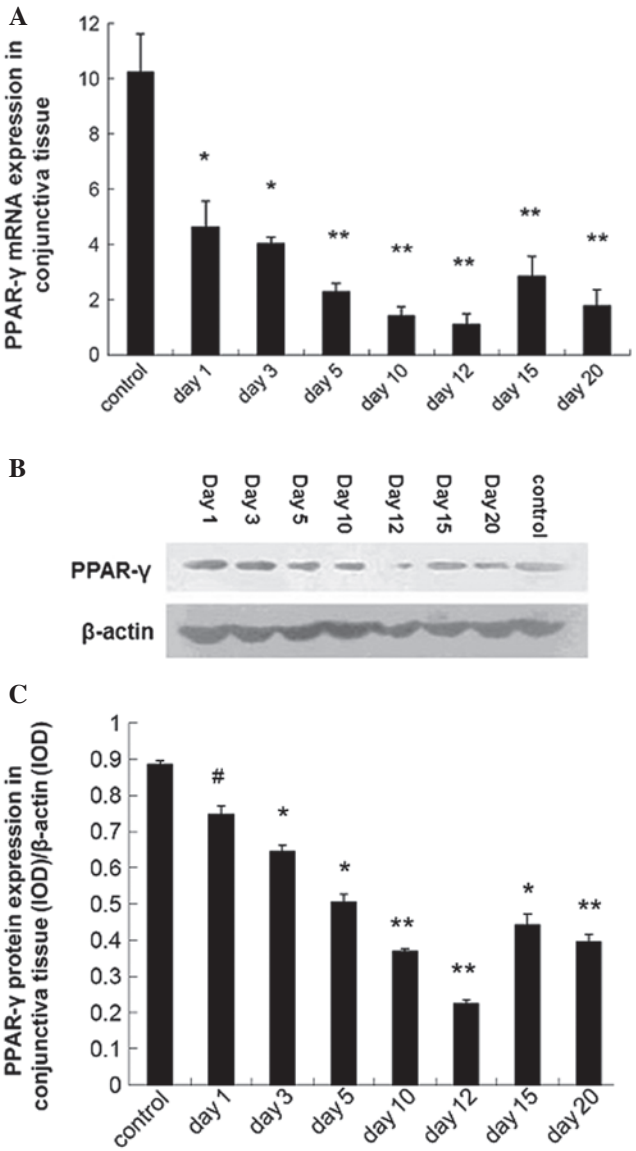

Figure 1. (A) Peroxisome PPAR-γ mRNA expression in the conjunctiva of dry eye mice and control mice. (B) Western blot analysis of PPAR-γ protein expression in the conjunctiva of dry eye mice and control mice. (C) Relative PPAR-γ protein expression to β-actin (n=3). #P<0.05, *P<0.01 and **P<0.001, vs. control. PPAR-γ, proliferator-activated receptor γ; IOD, integral optical density.

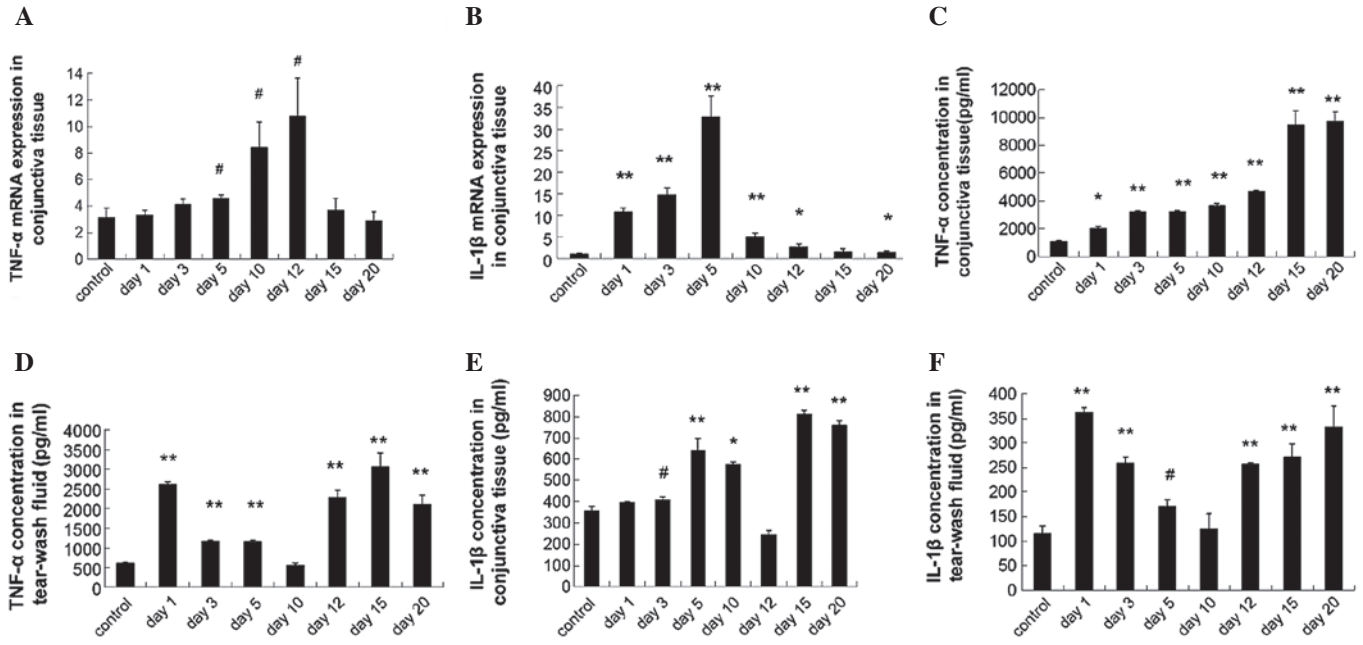

Figure 2. (A) TNF-α mRNA (B) IL-1β mRNA expression and (C) TNF-α concentration in the conjunctiva of dry eye mice and control mice. (D) TNF-α concentration of tear fluid (E) IL-1β concentration of the conjunctiva and (F) IL-1β concentration of tear fluid in control and dry eye mice (n=3). #P<0.05, *P<0.01 and **P<0.001 vs. control. TNF, tumor necrosis factor; IL, interleukin.

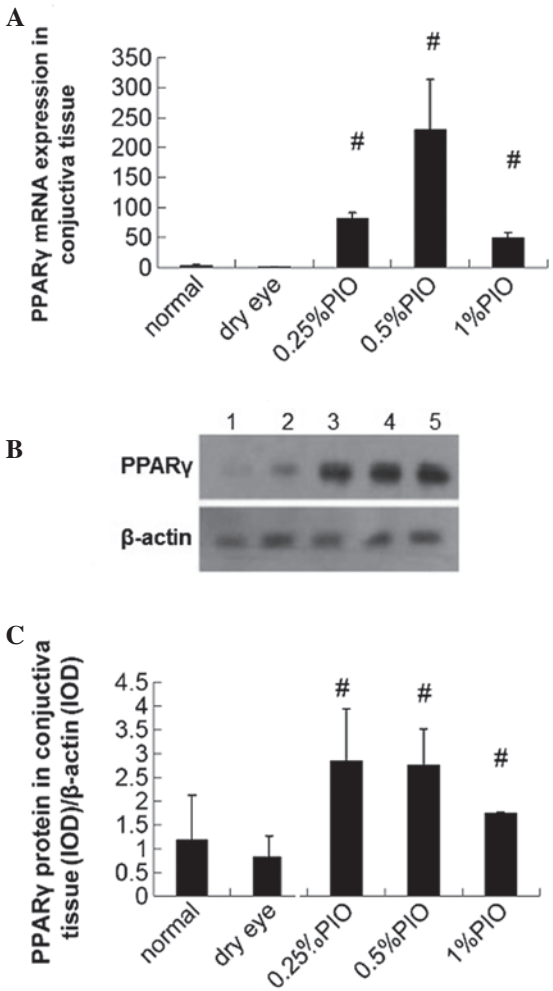

Figure 3. PPAR-γ mRNA and protein expression in different groups. 1, control; 2, dry eye; 3, 0.25% PIO; 4, 0.5% PIO and 5, 1% PIO. #P<0.05 vs. dry eye group. PPAR-γ, proliferator-activated receptor γ; PIO, pioglitazone.

Table III. Conjunctival goblet cell count of mice in different groups.

Group	Goblet cell count, n=5
Control	51.00±7.68
Dry eye	22.20±5.45
0.25% PIO	43.80±5.36[a]
0.5% PIO	48.20±5.40[a]
1% PIO	48.00±8.89[a]

[a]P<0.05 vs. the dry eye group. Magnification, x400.

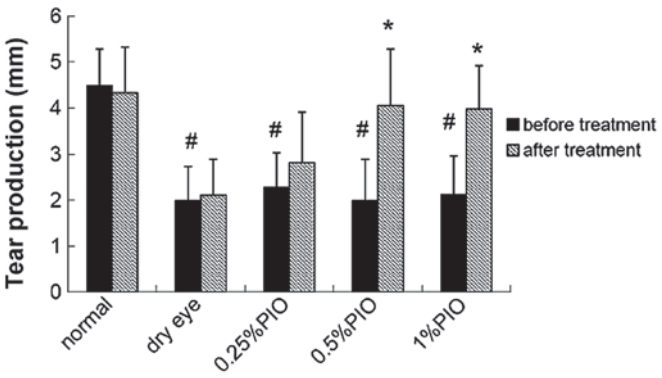

Figure 4. Tear production prior to and following induction of dry eye in different groups. #P<0.05, vs. control and *P<0.05, vs. the dry eye group.

639.33±59.14, 573.03±14.18, 243±19.58, 810.2±19.75 and 757.73±23.74 in dry groups and 353.6±26.36 in the control group (Fig. 2E). The TNF-α concentration in the tear fluid (days 1-20) was 362.37±9.26, 258.30±12.32, 171.10±13.08,

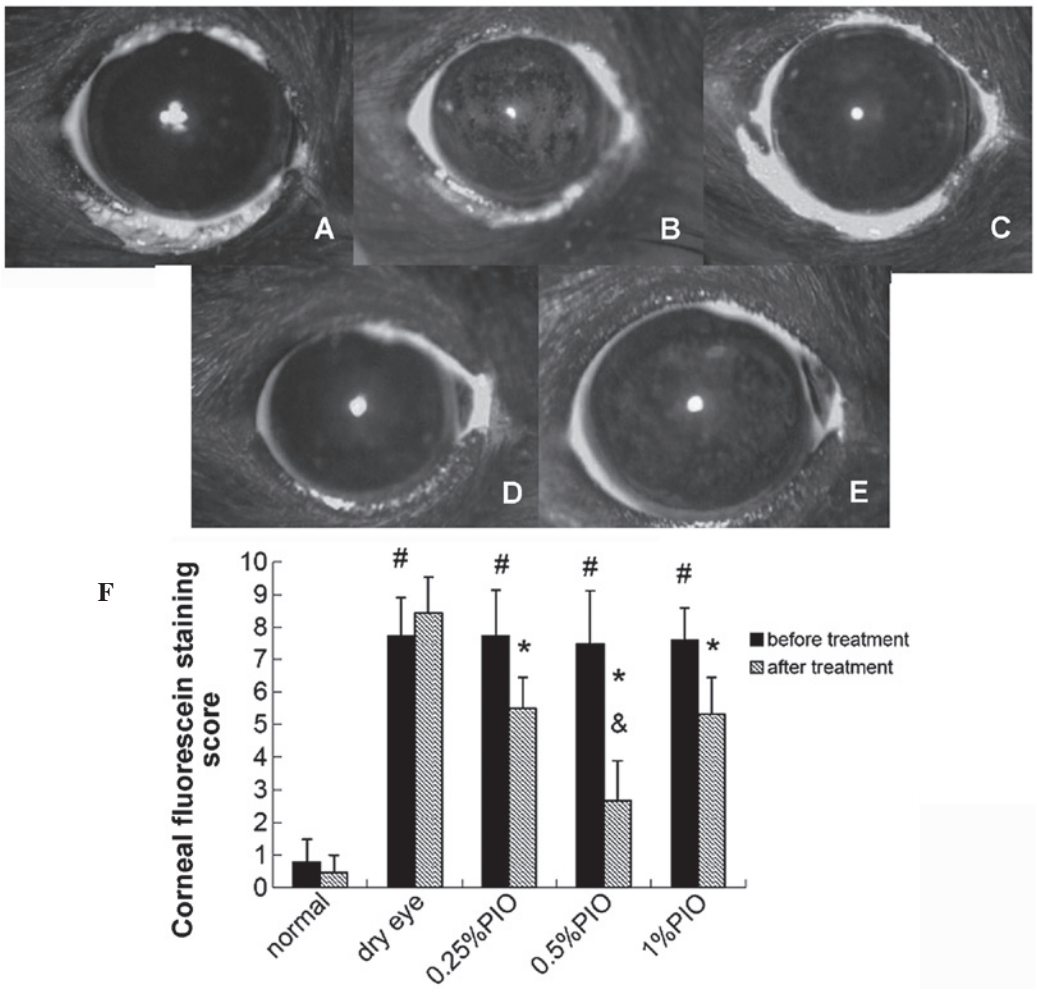

Figure 5. Corneal fluorescence staining in dry eye mice of different groups (slit lamp biomicroscope at cobalt blue light; magnification, x10). (A) Control, (B) dry eye, (C) 0.25% PIO; (D) 0.5% PIO and (E) 1% PIO groups. (F) Corneal fluorescence staining score in different groups. #P<0.05, vs. control; *P<0.05, vs. the dry eye group; and &P<0.05: 0.5% PIO, vs. the 0.25% PIO or 1% PIO groups. PIO, pioglitazone.

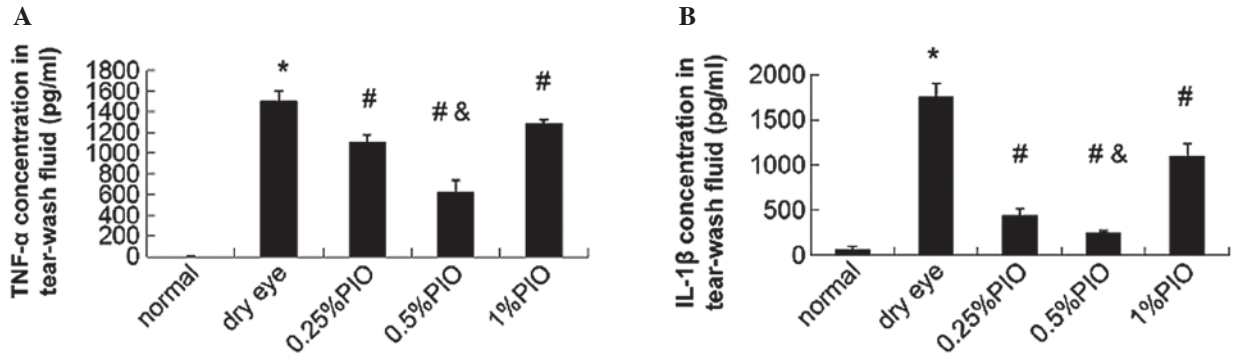

Figure 6. Contents of TNF-α and IL-1β in the tear fluid of different groups. *P<0.05, vs. control; #P<0.05, vs. the dry eye group; and &P<0.05: 0.5% PIO, vs. the 0.25% PIO or 1% PIO groups. TNF, tumor necrosis factor; IL, interleukin; PIO, pioglitazone.

124.17±31.93, 256.33±2.73, 270.67±26.53 and 332.17±42.88 in dry eye groups and 115.17±16.56 in the control group (Fig. 2F).

Influence of PIO treatment on dry eye. The PPAR-γ mRNA expression in dry eye groups was significantly lower compared with the control group; however, PIO treatment markedly increased the PPAR-γ mRNA expression when compared with the control and dry eye groups (P<0.05; Fig. 3A).

Western blot analysis revealed a weak band in the control and dry eye groups, suggesting a low PPAR-γ protein expression; however, the PPAR-γ protein expression increased markedly following PIO treatment. The PPAR-γ protein expression was normalized to β-actin protein expression, and results showed

the changes in the PPAR-γ protein expression were similar to those in PPAR-γ mRNA expression. In the dry eye groups, the PPAR-γ protein expression was significantly lower compared with the control group, however, the PIO treatment markedly increased the PPAR-γ protein expression as compared with the control group and dry eye groups (P<0.05; Fig. 3B and C).

Four weeks following PIO treatment, the tear production increased when compared with that of the dry eye group, and a significant difference was observed between the 0.5 and 1% PIO groups (P<0.05; Fig. 4).

Four weeks following the PIO treatment, the scores of corneal fluorescein staining were markedly lower compared with the dry eye groups (P<0.05), and significant differences were observed among the 0.5, 0.25 and 1% PIO groups (P<0.05; Fig. 5).

Four weeks following the PIO treatment, the number of goblet cells in the control and PIO groups was significantly higher compared with the dry eye group, and a significant difference was also noted between the PIO and dry eye groups (P<0.05; Table III).

In addition, the IL-1β and TNF-α concentrations of tear fluid in PIO groups were significantly lower compared with those in the dry eye groups (P<0.05; Fig. 6).

Discussion

In the present study, dry eye was induced by systemic administration of an anticholinergic agent, scopolamine, and exposure to a low humidity environment (12). Scopolamine is a competitive inhibitor of acetylcholine for muscarinic cholinergic receptors. Scopolamine-induced dry eye, which was demonstrated by a significant reduction in tear production and conjunctiva goblet cell count, and an increase in cornea fluorescein positive areas. These changes on the ocular surface are similar to those of human dry eye.

Dry eye is a complicated condition and its pathogenesis remains to be clearly elucidated. However, there is increasing evidence that dry eye is associated with ocular surface inflammation and relevant inflammatory cytokines. The initiation of the inflammatory response is associated with changes in local blood flow and accumulation of various inflammatory cells. Thus, the conjunctiva is hypothesized to be a crucial target at the early stages of inflammation, due to its rich blood supply. Notably, a large number of patients with dry eye present with lesions in the conjunctiva earlier than those in the cornea. The changes in the composition and hyperosmolarity of tear fluid and the dysfunction of the tear gland may promote inflammation of the ocular surface. Pflugfelder *et al* (15) reported that the mRNA expression of IL-1β, IL-6, IL-8, TNF-α and transforming growth factor-β1 increased significantly in the conjunctival epithelium of patients with Sjogren's syndrome when compared with healthy subjects. In non-Sjogren's dry eye patients, Yoon *et al* (16) also found similar findings. The present results showed that mice with dry eye presented with upregulated expression of pro-inflammatory cytokines in the conjunctiva, which was similar to the findings in the study of Luo *et al* (17) on scopolamine-treated mice. However, the current results showed that the expression of inflammatory cytokines increased following induction of dry eye. This may be partly due to the desiccated environment. The TNF-α mRNA expression increased in the conjunctiva on days 5, 10 and 12, and IL-1β mRNA expression was upregulated at all time points, with the exception of day 15. The concentration of inflammatory cytokines in the conjunctiva and tear fluid of dry eye mice was markedly higher compared with those in the control group. Notably however, the concentrations of TNF-α and IL-1β in the conjunctiva were significantly higher compared with those in the tear fluid. In the conjunctiva, inflammatory mediators are primarily released by local immune cells. The inflammatory cytokines in the tear fluid are mainly secreted by the lacrimal gland. This suggests that decreased lacrimal gland secretion is partly attributed to the development of dry eyes. However, in the conjunctiva, the response to decreased tear production and the stress of desiccation may initiate and trigger the inflammatory process.

There is an increasing body of evidence indicating that PPAR-γ and its activators are important in the regulation of inflammatory processes (18). A previous study showed that PPAR-γ is involved in the control of inflammation, particularly in the modulation of the production of a number of inflammatory mediators, including TNF-α and IL-1β (19). This is partly associated with the inhibited activation of NF-κB, which is an important transcription factor in the inflammatory response. PPAR-γ interferes with inflammatory pathways, including the NF-κB pathway by interacting with p50 and p65 *in vitro* (20). In the present study, the results showed that the mRNA expression of PPAR-γ in the conjunctiva of dry eye mice was downregulated significantly one day following dry eye induction. Although the PPAR-γ expression reduced, its expression was significantly different to that in the control group. The PPAR-γ protein expression in the conjunctiva showed a similar trend to the PPAR-γ mRNA expression. This suggests that decreased tear production and the desiccated environment may inhibit PPAR-γ activity. Notably, these results were closely associated with the features of dry eye mice in terms of aqueous tear production, corneal fluorescein staining scale and goblet cell count of the conjunctiva. By contrast, the expression of TNF-α and IL-1β increased to different extents. It is worth noting that, although the mRNA expression of TNF-α was not fully consistent with the changes in PPAR-γ expression, the TNF-α protein expression in the conjunctiva was consistent with the trend of the changes in PPAR-γ expression. In the tear fluid, the TNF-α content of dry eye mice at different time points was higher compared with that in the control group. This suggests that, in the development of dry eyes, the conjunctival inflammation and the changes on the ocular surface were associated with the changes in PPAR-γ expression. It has been suggested by Gao *et al* (21) that TNF-α may inhibit the PPAR-γ activity at two different levels. The mRNA expression of PPAR-γ may be downregulated by TNF-α, which was confirmed by Ruan *et al* (22). By contrast, it has been suggested that extracellular signal-regulated kinase and c-JUN NH2 terminal kinase in the TNF-α signaling pathway may inhibit the transcriptional activity of PPAR-γ through phosphorylating serine residues of PPAR-γ and may suppress ligand-dependent PPAR-γ activity without decreasing PPAR-γ expression (23). Thus, PPAR-γ may negatively modulate the production of inflammatory cytokines, including TNF-α, and by contrast, these components may interfere with

the PPAR-γ activity. However, the exact mechanism requires further investigation.

PPAR-γ has two types of ligands, namely endogenous ligands and chemically synthesized ligands. The endogenous ligands are mainly composed of polyunsaturated fatty acids and fatty acid derivatives, and the chemically synthesized ligands, including diketones thiazolidines (rosiglitazone, pioglitazone) and tyrosine derivatives (GW7845). Natural agonists of PPAR-γ usually have poor specificity and chemically synthesized agonists present with relatively high specificity. Glitazones are a group of derives with a thiazolidinedione structure. Studies have shown that glitazones may selectively activate PPAR-γ to increase the peripheral sensitivity to insulin and reduce the blood glucose level in patients with type 2 diabetes. In previous years, increasing numbers of studies have been conducted to investigate the roles of PPAR-γ in the regulation of inflammation, immunity and cell proliferation, and PIO has been used in the treatment of inflammatory diseases and tumors. There is evidence showing that PIO may inhibit the secretion of inflammatory cytokines, including TNF-α and IL-6, by macrophages to improve the symptoms of asthma patients. In addition, PIO has been obseved to inhibit the release of MMP-9 (a pro-inflammatory cytokine) lipid peroxidation and accumulation of polymorphonuclear leukocytes to improve ischemia/reperfusion-induced lung injury. PIO can also inhibit vascular endothelial adhesion molecule expression and interfere with the inflammatory response to alleviate ulcerative colitis; PIO may inhibit the production of IL-1β, TNF-α, COX-2 and iNOS in the ulcerative mucosa and blood and increase heat shock protein 70 production to promote gastric ulcer healing (24-26).

In previous years, PIO has been used in the treatment of corneal neovascularization and diabetic retinopathy (10,27,28). Traditionally, PIO is used by intravitreal injection or subconjunctival injection. However, these injections are invasive and not suitable for the treatment of dry eye. Thus, PIO drops are more suitable in the treatment of eye diseases. PIO has a small molecular weight, is fat-soluble (insoluble in water but soluble in organic solvents, including ethanol and acetonitrile) and has a low toxicity. In addition, the pH value may be adjusted to be neutral. Thus, it is proposed that PIO has good corneal permeability. In this study, results showed that following treatment with 0.25, 0.5 or 1% of PIO for 4 weeks, the PPAR-γ expression increased markedly when compared with healthy mice and dry eye mice, and the PPAR-γ mRNA and protein expression showed a similar tendency. This suggests that PIO drops may be absorbed by the ocular surface and may activate PPAR-γ expression on the ocular surface. Detection of PPAR-γ expression under different conditions may be used to evaluate the inflammation in dry eye conditions, which may be beneficial for the investigation of the role of PPAR-γ in inflammatory regulation and elucidation of the clinical significance of PIO.

Following treatment with PIO drops, the PPAR-γ expression in the conjunctiva increased, accompanied by an increase in tear production, improvement of corneal fluorescein staining scores, elevation of goblet cell count, reduction in TNF-α and IL-1β concentration in the tear fluid and improvement of conjunctival pathology when compared with dry eye mice without treatment. This demonstrates that upregulated PPAR-γ expression may reduce the expression of inflammatory cytokines to inhibit the inflammatory process, increase the tear production, improve the tear film stability and attenuate the damage to the ocular surface, which then exerts therapeutic effects on dry eyes.

In the present study, PIO was used at three concentrations, showing differences in the therapeutic efficacy. Although the symptoms of dry eye and the damage to the ocular surface were improved to different extents, the improvement of corneal fluorescein staining scores was the most evident in the 0.5% PIO group ($P<0.05$, vs. 0.25% PIO group and 1% PIO group). In addition, TNF-α and IL-1β expression was the lowest in the 0.5% PIO group followed by the 0.25% PIO group and highest in the 1% PIO group. The PPAR-γ mRNA expression was highest in the 0.5% PIO group and lowest in the 1% PIO group. The PPAR-γ protein expression was comparable between 0.5% PIO and 0.25% PIO groups, however, the PPAR-γ protein expression in the former two groups was significantly higher compared with the 1% PIO group. Results showed the therapeutic efficacy and stability of 0.25% PIO and 1% PIO are inferior to those of 0.5% PIO. Thus, 0.5% is hypothesized as an ideal concentration of PIO for the treatment of dry eye. However, few studies have been conducted to investigate the therapeutic efficacy of PIO drops. Thus, the concentration, safety and side effects of PIO drops require further elucidation.

In conclusion, the results showed the ocular manifestations of mice with dry eye. The increased expression of inflammatory cytokines, including TNF-α and IL-1β in the conjunctiva and tear fluid may be associated with the downregulation of PPAR-γ expression on the ocular surface. However, the exact mechanism underlying the changes in PPAR-γ expression during the development of dry eye is poorly understood. We hypothesize that there is an interaction between PPAR-γ and inflammatory mediators.

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